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| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | 12b. DISTRIBUTION CODE |
| 13. Abstract (Maximum 200 Words) (<i>abstract should contain no proprietary or confidential information</i>) Retinoids, derivatives of vitamin A (retinol), are required for the appropriate differentiation of normal human prostate epithelial cells. Human prostate cancer cells contain much lower levels of vitamin A and its metabolites than normal cells. We hypothesize that aberrant metabolism of vitamin A and dysregulation of gene expression in prostate tumor cells are related to the abnormal growth properties of the tumor cells. A rationale for using retinoids in prostate cancer chemotherapy is further supported by the effectiveness of ATRA (all-trans Retinoic Acid), a vitamin A metabolite, in the treatment of acute promyelocytic leukemia (APL). We hypothesize that the efficacy of retinoic acid can be enhanced if it is administered in combination with low doses of selective, potent histone deacetylase inhibitors such as trichostatin A (TSA) or valproic acid. The goals of this idea grant are to use mouse xenograft models to ascertain the effectiveness of various retinoids plus histone deacetylase inhibitors in inhibiting the growth and inducing the differentiation of the human prostate cancer lines LNCaP and PC-3. A second goal of the project is to understand at the molecular level the mechanisms by which the combination of retinoic acid and histone deacetylase inhibitors result in human prostate tumor cell growth inhibition. In the past year we have performed a variety of biochemical and molecular biological assays on human prostate cancer cells treated with various combinations of the aforementioned drugs in order to gain more insight into the molecular mechanisms involved in cell growth inhibition. The studies that we have performed, and the studies proposed in the next period of this Idea Development grant should provide a much clearer rationale for new clinical treatments for prostate cancer in humans. | | | |
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INTRODUCTION

Retinoids such as retinoic acid have been used for a number of years to treat a variety of cancers. For instance, retinoic acid has been used successfully in the treatment of acute promyelocytic leukemia (APL). While ATRA (all-trans retinoic acid) administration results in remissions in most APL patients and the subsequent cures of the patients following secondary, cytotoxic cancer chemotherapy, occasional cases of patient relapse occur and the leukemia is then sometimes ATRA-resistant. In such patients, the addition of a histone deacetylase inhibitor, such as sodium phenylbutyrate, induced a complete molecular remission. The mechanism for the increased efficacy of retinoic acid in the presence of histone deacetylase inhibitors most likely involves the ability of histone deacetylase inhibitors to drive the formation of a more active transcription complex involving the retinoic acid receptors, retinoid X receptors, histone acetyltransferases, and coactivator proteins (for rev., ref. (1)).

The clinical data from the acute promyelocytic leukemia patients, plus data generated in our laboratory, led us to develop the hypothesis that the combination of retinoids with histone deacetylase inhibitors should more effectively bring about cell differentiation and the control of cell growth in other types of tumors in addition to acute promyelocytic leukemia. More specifically, we hypothesize that cell differentiation is dysregulated in prostate cancer and that treatment of prostate cancer cells with pharmacological doses of all-trans-retinoic acid plus an inhibitor of histone deacetylase will result in greater tumor growth inhibition and tumor cell differentiation than treatment with either all-trans-retinoic acid or a histone deacetylase (HDAC) inhibitor alone. We propose to test aspects of this model in a more clinical setting, using a human prostate cancer xenograft model in Nu/Nu mice, and to test mechanistic aspects of this hypothesis using cultured human prostate cancer cell lines.

BODY

Task 1. To determine the mechanism by which concomitant administration of retinoids and various histone deacetylase inhibitors such as trichostatin A (TSA) inhibits human prostate cell cancer growth (months 1-36).

CELL PROLIFERATION INHIBITION

In task 1, we employed cell proliferation assays to determine if we observed increased growth inhibition using combination therapy with ATRA plus a low dose of a variety of different histone deacetylase inhibitors compared to either drug alone or to the untreated, control tumor cells. Both LNCaP and PC-3 human prostate cancer cell lines were tested. Various retinoids were tested, including 13-cis-retinoic acid, all-trans-retinoic acid, and 9-cis-retinoic acid, since all of these retinoids have been successfully used in the treatment or chemoprevention of several different human cancers. The histone deacetylase inhibitors that were tested included trichostatin A, valproic acid, and SAHA (suberoylanilide hydroxamic acid (SAHA)) (2-5). We utilized several histone deacetylase inhibitors because a number of these inhibitors are new, but positive results have been obtained in mouse models and for some, in human cancer therapy trials (2-7). We also added the drug 5-aza-deoxycytidine to our growth inhibition studies because of

much recent data that in combination with histone deacetylase inhibitors, the addition of 5-aza-CdR treatment results in demethylation and enhanced gene expression (8-10).

Our data, some of which are shown in this report, indicate that for LNCaP, while both valproic acid (VPA) and trichostatin A plus ATRA or retinol were growth inhibitory, VPA plus ATRA or retinol was more growth inhibitory than was TSA plus ATRA or retinol (Figure 1). The drugs 13-cisRA and 9-cisRA also resulted in growth inhibition in combination with VPA (data not shown).

In contrast, for the PC-3 cell line trichostatin A plus RA or retinol was more growth inhibitory than was VPA plus ATRA or retinol (Figure 2). Furthermore, the addition of aza-deoxycytidine to either VPA plus retinol or VPA plus retinoic acid led to enhanced growth inhibition of the PC-3 cells (Figure 3).

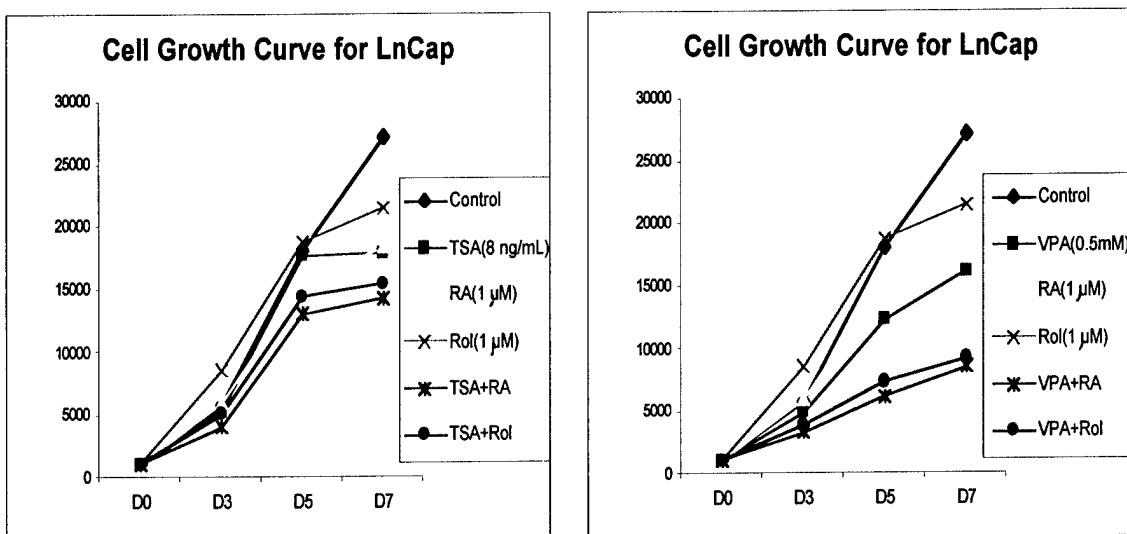


Figure 1A, B. The inhibitory effects of retinoic acid (RA, 1 µM) and retinol (Rol, 1 µM) on human LNCaP prostate cancer cells in combination with the histone deacetylase inhibitor trichostatin A (TSA, 8 ng/ml) or valproic acid (VPA, 0.5 mM). The LNCaP cells (1×10^6 /well) were plated in 24-well cell plates for 16 hours before the indicated drugs were administered. The cells were trypsinized and counted using a Coulter counter in triplicate after incubation with the drugs for 3, 5, or 7 days.

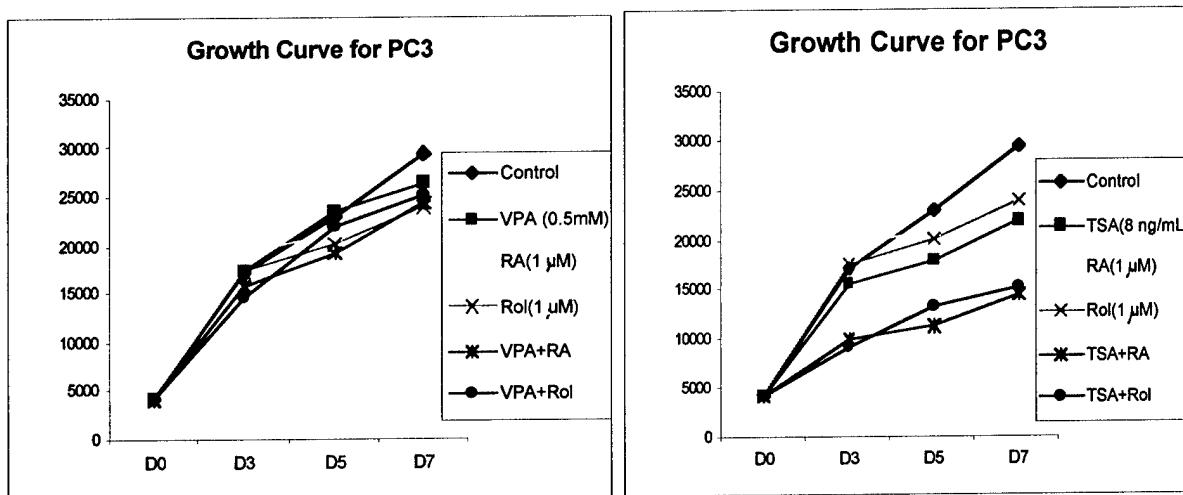


Figure 2A, B. The inhibitory effects of all-trans retinoic acid (RA, 1 μ M) or retinol (Rol, 1 μ M) on human PC-3 prostate cancer cells in combination with the histone deacetylase inhibitor trichostatin A (TSA, 8 ng/ml) or valproic acid (VPA, 0.5 mM). The PC-3 cells (3×10^6 /well) were plated in 24-well cell plates for 16 hours before the indicated drugs were administered. The cells were trypsinized and counted in triplicate using a Coulter counter after incubation with the drugs for 3, 5, or 7 days.

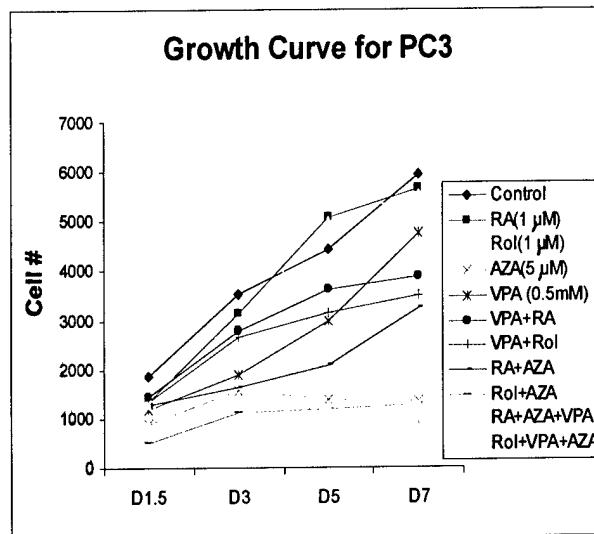


Figure 3. The inhibitory effects of retinoic acid (RA, 1 μ M) or retinol (Rol, 1 μ M) on human PC-3 prostate cancer cells in combination with the histone deacetylase inhibitor valproic acid (VPA, 0.5 mM) plus the DNA methyltransferase inhibitor 5-aza-deoxycytidine (5 μ M). The PC-3 cells (3×10^6 /well) were plated in 24-well cell plates for 16 hours before the indicated drugs were administered. The cells were trypsinized and counted in triplicate using a Coulter counter after incubation with the drugs for 1.5, 3, 5, or 7 days.

We conclude from these growth inhibition assays that a combination of a retinoid, histone deacetylase inhibitor, and a demethylating agent such as aza-deoxycytidine is most efficacious at inhibiting the growth of prostate cancer cells in culture. Additionally, one very intriguing observation from our studies is that different HDAC inhibitors, in combination with retinoids such as retinoic acid or retinol, inhibit the growth of different prostate cancer cell lines to varying degrees, i.e. there is a cancer cell specificity to the HDAC inhibitors. It is known that there are different classes of HDAC inhibitors (reference 2 for review) but the roles of the various histone deacetylases (currently 11 different HDACs are known) in cells and the levels of expression of the various histone deacetylases in cells are not well understood. Thus, one conclusion from our data is that different HDAC inhibitors may have roles to play in the chemotherapy of a variety of different types of cancers and molecular subtypes of prostate cancers.

EXAMINATION OF MOLECULAR MARKERS OF CELL DIFFERENTIATION IN CULTURED PROSTATE CELLS BY NORTHERN ANALYSIS, RT-PCR, AND WESTERN ANALYSES.

We have examined a number of different genes which are expressed at higher levels in differentiated, normal human prostate epithelial cells vs. prostate cancer cells. Untreated cells, as compared to cells treated with various combinations of retinoids plus HDAC inhibitors, were examined. For example, we have measured the levels of the keratin 18 and keratin 8 genes in these cells by Northern analysis. We did not detect changes in keratin 8 or 18 mRNAs in response to the combination of HDAC inhibitors plus retinol or retinoic acid treatment. However, we did detect a three to five fold increase in keratin 8 and keratin 18 mRNA levels in both cultured normal prostate epithelial cells and in prostate cancer cell lines treated with retinoic acid or retinol (1×10^{-7} M) alone. Cell cycle analysis indicated that the cells treated with retinoids plus HDAC inhibitors arrested in the G1/G0 phase of the cell cycle. Further studies of molecular markers of cell proliferation inhibition and cell differentiation are planned in months 12-24.

Task 2. In task 2, we propose to evaluate the efficacy of combined retinoid/histone deacetylase inhibitor administration in xenograft models (months 4 through 36). We have begun these experiments, but we do not have reportable data at the present time. The goal of task 2 is to establish a human prostate tumor xenograft model in Swiss nude, immunocompromised mice. Drugs are then administered in four experimental arms, including control, retinoid alone, histone deacetylase inhibitor alone, and the combination of retinoid and histone deacetylase inhibitor in combination. The tumor growth is monitored by measuring tumor area and molecular markers of apoptosis, cell growth, and cell differentiation are to be assessed. RNA and protein from the tumors will be isolated for assessment, and both differentiation specific and pro-apoptotic genes will be measured by Northern and Western blotting. Tissue slides will be prepared and stained with hematoxylin and eosin for histological characterization of the tumors, both untreated and drug treated.

We are now in the process of performing these experiments. As the xenograft models are complicated, expensive, and require large numbers of Nu/Nu

immunocompromised mice, we have had to carry out appropriate training of the researchers involved. As a result, we did not begin these experiments until month 9.

KEY RESEARCH ACCOMPLISHMENTS

- A. The demonstration that in *different* prostate tumor lines various histone deacetylase inhibitors result in different degrees of cell growth inhibition when combined with retinoids such as all-trans retinoic acid or retinol.
- B. The demonstration that 5-aza-deoxycytidine, in combination with a retinoid and a histone deacetylase inhibitor, results in a greater degree of growth inhibition in human prostate cancer cell lines.
- C. The analysis of a variety of molecular markers such as keratin 8, keratin 18, and PSA (prostate specific antigen) expression in both normal human prostate epithelial cells and human prostate cancer cells cultured either as control cells, or in the presence of a variety of combinations of retinoids plus histone deacetylase inhibitors.
- D. The measurements of acetylated histones, retinoid receptors, and other molecular markers of cell growth inhibition and apoptosis induction in cultured human prostate cancer cell lines following treatment with retinoids plus histone deacetylase inhibitors.

REPORTABLE OUTCOMES

Touma, S.E., Masterton, J., Navarro, D., Nanus, D., Tickoo, S., and Gudas, L.J. Analysis of gene expression in cultured human prostate cancer cells following treatment with various retinoids and histone deacetylase inhibitors. In preparation.

CONCLUSIONS

First, we have shown that there are differences in the responses of various human prostate cancer cell lines to different classes of histone deacetylase inhibitors. For example, the combination of the drug valproic acid plus retinoic acid was more growth inhibitory to LNCaP cells than was SAHA or trichostatin A plus all-trans retinoic acid. In contrast, trichostatin A plus retinoic acid was much more growth inhibitory to PC-3 cells than was VPA plus all-trans retinoic acid. Such findings could have significant clinical applications. The LNCaP line is androgen responsive, whereas the PC-3 line is not. Furthermore, these data suggest that the examination of the different histone deacetylases expressed in various tumor lines could be worthwhile. Second, we have preliminary data that the combination of a retinoid and a histone deacetylase inhibitor results in growth inhibition and there is also some apoptosis of the cells. We did not find evidence for increased differentiation of the tumor cells in response to retinoids plus histone deacetylase inhibitors, but retinoids alone caused an increase in some differentiation markers in the prostate cancer cell lines. Third, we have shown for the first time that valproic acid alone results in some growth inhibition of the LNCaP prostate cancer cell line.

In addition to gaining fundamental knowledge of the mechanisms of action of retinoids plus histone deacetylase inhibitors in combination, our studies should provide insights for future pharmacological therapies for human prostate cancer treatment.

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